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# Folate status assessment history: implications for measurement of biomarkers in NHANES<sup>1-5</sup>

Barry Shane

## ABSTRACT

This article presents a historical perspective on the different methods used to measure folate status in populations and clinical settings. I discuss some of the advantages and limitations of these procedures. For >50 y researchers have used microbiological assay methods to assess folate status in clinical settings and in population-based studies, such as NHANES. Serum and red blood cell folate values obtained with the *Lactobacillus casei* assay have formed the basis for current ranges and cutoffs for the establishment of folate sufficiency and for the current dietary reference intakes for folate. Over the past 30 y competitive folate protein binding assays, which are available in kit form, have supplanted microbiological assays in many clinical laboratories because of their ease of use. Several NHANES cycles have used these assays. Folate concentrations obtained with these kits are lower than those from microbiological assays and show a wide variation between different protein binding assay kits. This variation has complicated the setting of values for normal ranges of folate status and the comparison of status changes between different NHANES cycles. The recent development of mass spectrometry methods for folate opens up the possibility of measurement of individual folate vitamers such as folic acid. Past experience with microbiological and competitive protein binding assays indicates some of the technical problems that research will need to address before this promise becomes reality. *Am J Clin Nutr* doi: 10.3945/ajcn.111.013367.

## INTRODUCTION

Folate is the group name for a class of bioactive vitamin compounds that are based on the folic acid structure and that are interconverted during metabolism (1–3). A large number of potential folate derivatives exist that differ in oxidation state, one-carbon substitution, and polyglutamate chain length (**Figure 1**). Folic acid itself does not occur naturally and is usually present only in individuals who take vitamin supplements or eat fortified foods. Reduced folates are usually less stable than folic acid and their stability varies, depending on the one-carbon substitution. The oxidation of reduced folates usually results in products that lack vitamin activity, although a small proportion may be converted to biologically active oxidized forms.

The large number of folate derivatives, and the instability of some of them, as well as the potential for some of them to interconvert chemically after extraction from biological samples, greatly complicates the identification and assaying of individual folate vitamers. Historically, most of the assay methods that have been used to measure folate status have measured total folate,

primarily in serum, plasma, or red blood cells. Microbiological assay methods, which were originally developed in the 1930s, were the first method used to measure folate status and were the “gold standard” for many years. Many laboratories still use these methods. Over the past 30 y, assays based on competitive protein binding have become more popular, particularly in clinical laboratories, because of their ease of use and availability in commercial kit form. More recently, a number of laboratories have introduced mass spectrometry methods that have the potential to measure individual folate one-carbon forms.

Plasma contains only folate monoglutamates, almost entirely the 5-methyltetrahydrofolate (5-methyl-THF) form, whereas long-chain polyglutamates of 5-methyl-THF predominate in red blood cells. In recent years, interest has increased in the assay of individual folate one-carbon forms. This increased interest is partly because of the observation that common genetic polymorphisms, such as the 677C→T variant in the methylenetetrahydrofolate reductase gene (5), may cause a redistribution of folate one-carbon forms in red blood cells and possibly other tissues (6). In addition, the introduction of folic acid fortification of the food supply, as well as folic acid supplement use, has resulted in the appearance of free folic acid in blood samples, and some concerns have been raised that the presence of this unnatural form of folate may have detrimental effects (7).

## MICROBIOLOGICAL ASSAY METHODS

The introduction in the 1930s of microbiological assay methods that used lactic acid bacteria for the detection and assaying of vitamin factors greatly aided in the discovery, isolation, and characterization of many B vitamins. These microbiological assay

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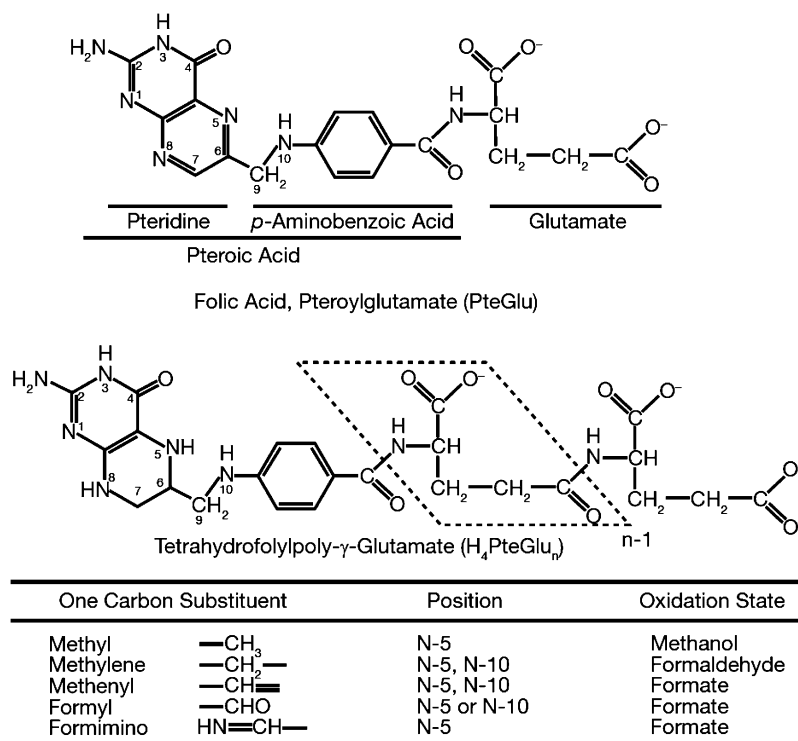


FIGURE 1. Structure of folic acid and reduced folylpolyglutamates. Reproduced with permission from reference 4.

methods represented a marked improvement over other bioassay methods, such as chick growth bioassays, that laboratories used at that time (8). Most bacteria synthesize folate *de novo* (as dihydrofolate) and cannot transport folate. Bacteria use folate to synthesize thymidylate, purines, methionine, serine, glycine, and pantothenate. Bacteria also use folate for <sup>f-met</sup>tRNA synthesis, which is necessary for protein synthesis initiation. Lactic acid bacteria are usually present in the gut and have lost the ability to synthesize many of the B vitamins and survive in their environment because they have developed transport systems for various B vitamins (9, 10).

Folate was originally isolated in the 1940s from spinach, liver extracts, and *Corynebacterium* species cultures as a factor that supported *Lactobacillus casei* (renamed *Lactobacillus rhamnosus*, ATCC 7469) growth (11, 12). A major advantage of assays with *L. casei* is that *L. casei* responds equally well to all folate monoglutamate forms (Table 1) (8). Although the affinity for the folate transporter varies with different monoglutamates, growth promotion is essentially identical because intracellular one-carbon metabolism, rather than transport, limits growth on monoglutamates (13, 14).

The authors of one study (15) reported that 5-methyl-THF produced a significantly decreased growth response compared with folic acid when the medium's pH was 6.8, whereas responses were identical at a pH of 6.2. The pH of 6.2 is the one that researchers have traditionally used for assays with this microorganism.

*L. casei* possesses a vitamin B-12-independent polyglutamate-specific methionine synthase. As a result, the initial metabolism of transported 5-methyl-THF involves its oxidation to 5,10-methylene-THF via a NAD-dependent methylenetetrahydrofolate reductase (13). Growth of *L. casei* on folate diglutamates is essentially identical to that on monoglutamates. Growth of *L. casei* on triglutamates is similar but may be slightly lower.

*L. casei* is unusual in that it can also transport long-chain folylpolyglutamates, although the affinity for the transporter decreases with elongation of the glutamate chain (16, 17). Long-chain folylpolyglutamates are transported intact and used directly in one-carbon metabolism, but with these compounds transport becomes limiting for growth. As a result, the growth curves obtained in response to folate concentration with polyglutamates of chain length >3 are steeper (less exponential) than those obtained with folylmono- to triglutamates (16). This phenomenon, called positive drift, prevents the normalization of growth curves obtained with long-chain polyglutamates to the curves from shorter derivatives.

Folylpolyglutamates in biological samples such as red blood cells require hydrolysis to the triglutamate or shorter glutamate chain length for an accurate assay of total folate by *L. casei*. Because folate concentrations in plasma (entirely monoglutamate) are much lower than those in red blood cells, hydrolysis has normally been achieved by the lysing of whole blood and incubation of the lysate for 1–2 h, which allows hydrolysis of folylpolyglutamates by a neutral pH plasma  $\gamma$ -glutamyl hydrolase.

In some studies, investigators treated red blood cell extracts with hog kidney conjugase, which converts folates to monoglutamates under acidic conditions, or with chick pancreas conjugase, which converts folates to the diglutamate form. Hydrolysis of folylpolyglutamates by these enzymes proceeds rapidly with the long-chain derivatives but slows down with shorter polyglutamates, such that the terminal hydrolysis (usually, the conversion of diglutamate to monoglutamate) is relatively slow and can be incomplete. Because *L. casei* responds well to di- and triglutamates, incomplete hydrolysis of folylpolyglutamates to the monoglutamate is not normally a concern.

**TABLE 1**  
Relative response of microorganisms to unhydrolyzed folate derivatives<sup>1</sup>

	<i>Lactobacillus casei</i>	<i>Streptococcus faecium</i>	<i>Pediococcus cerevisiae</i>
Folic acid (PteGlu)	96	100	0
10-Formyl-PteGlu	100	100	0
Dihydrofolate	100	100	0
THF	100	100	100
10-Formyl-THF	99	100	100
5-Formyl-THF	100	100	100
5-Methyl-THF	100	0	0
PteGlu <sub>2</sub>	100	100	0
PteGlu <sub>3</sub>	100	0	0
PteGlu <sub>4</sub>	66	—	—
PteGlu <sub>5</sub>	20	—	—
PteGlu <sub>6</sub>	4	—	—
PteGlu <sub>7</sub>	2	—	—
10-Formyl-H <sub>4</sub> PteGlu <sub>3</sub>	82	—	—
10-Formyl-H <sub>4</sub> PteGlu <sub>5</sub>	3	—	—
5-Methyl-H <sub>4</sub> PteGlu <sub>3</sub>	90	0	0
5-Methyl-H <sub>4</sub> PteGlu <sub>5</sub>	2	0	0
5-Methyl-H <sub>4</sub> PteGlu <sub>7</sub>	1	—	—
Pterate	0	100	0
10-Formyl-pterate	0	100	0
5-Formyl-H <sub>4</sub> pterate	0	+	0

<sup>1</sup> In most cases, the relative response is a comparison of folate concentration required to support half maximum growth rates of the bacterium. The data were adapted from reference 9. All reduced folates refer to the natural isomer at the C-6 position. 6R,S-5-Methyl-THF and 6R,S-5-formyl-THF, which researchers sometimes use as standards, exhibit half the activity of the 6S-derivatives that correspond to them. THF, tetrahydrofolate; +, some growth but not quantitated.

The authors of many studies in the literature, particularly in the food science area, have used the terms “free” and “total” folate to denote the folate concentrations that result from assays with *L. casei* before and after  $\gamma$ -glutamyl hydrolase treatment. These authors assumed that “free” folate refers to folate monoglutamates and short-chain polyglutamates. However, this greatly exaggerates the amount of short-chain derivatives due to *L. casei*'s ability to respond to longer-chain folypolyglutamates.

*L. casei* exhibits a growth response not only to folate but also to products of one-carbon metabolism, and grows in the complete absence of folate if the medium contains thymidine, purines, methionine, serine, glycine, and pantothenate (14). Human plasma contains significant concentrations of all of these compounds, with the exception of thymidine. For this reason, *L. casei* media for the folate assay contain amino acids, purines, and pantothenate. Under these conditions, the growth of *L. casei* in response to folate is due to the organism's folate requirement for thymidylate synthesis.

## DIFFERENTIAL MICROBIOLOGICAL ASSAY

The types of folate that support growth of other bacteria can differ from that observed with *L. casei*, and these differences in folate requirement specificity have been used to assess the amounts of different folate one-carbon forms in biological extracts (9). *L. casei* responds to all one-carbon forms of folate as well as to folic acid, dihydrofolate, and THF. *Streptococcus faecium* (formerly known as *Streptococcus faecalis* or *Strepto-*

*coccus lactis R*) also requires folate for growth and responds to most folate mono- and diglutamates (Table 1). It differs from *L. casei* in its inability to grow on tri- or longer polyglutamates and its inability to use 5-methyl-THF for growth. 5-Methyl-THF is transported by *S. faecium* but it cannot metabolize it.

Researchers have used the growth response of *S. faecium* to measure the amount of non-methyl-folate in biological extracts and the amount of 5-methyl-THF, which they calculated by subtracting the *S. faecium* value from the value they obtained for total folate with the use of the *L. casei* assay (reviewed in reference 9). *S. faecium* also differs from *L. casei* in that it responds to pterate derivatives because it possesses dihydrofolate synthetase activity, which *L. casei* lacks. Pterates have no folate activity in humans, who also lack dihydrofolate synthase activity. It is not known whether any pterates are present in human biological samples, such as plasma.

The folate requirement of *Pediococcus cerevisiae* (formerly known as *Leuconostoc citrovorum*) has been less well characterized (Table 1) (9). This organism is similar to *S. faecium* in that it responds to all reduced folate monoglutamates with the exception of 5-methyl-THF. *P. cerevisiae* differs from *S. faecium* in that folic acid and dihydrofolate, and also 10-formyl folic acid, which can arise through oxidation of reduced folates, do not support its growth. Consequently, a reduced folate, normally 5-formyl-THF, rather than folic acid has to be used to generate the standard curve when this organism is used for microbiological assay procedures. Differences in folate concentrations obtained after assay with *S. faecium* and *P. cerevisiae* have been used to calculate the folic acid content of biological samples. However, this “folic acid” content could also reflect the presence of dihydrofolate and/or 10-formyl folic acid in the sample.

An additional drawback in the use of these organisms for differential microbiological assays of blood samples for routine folate status assessment relates primarily to the accuracy and precision of the assays. Assays with *S. faecium* can provide an assessment of total non-methyl forms of folate in plasma or red blood cells but not individual folate one-carbon forms. Measurement of total folate still requires assays with *L. casei* because most of the folate in these samples is in the form of 5-methyl-THF.

Comparison of growth responses between *S. faecium* and *P. cerevisiae* may supply information on free folic acid content. However, because the concentrations of folic acid and non-methyl folate are typically very low, a comparison of growth responses would require very high precision and accuracy to obtain an accurate measure of folic acid by difference. In any event, the presence of other oxidized derivatives could confound the results. Although the microbiological methods are very sensitive, it is not clear that they possess sufficient accuracy and precision for routine assessment of minor folate species. The routine use of 3 different microorganisms is also quite cumbersome.

The *L. casei* microbiological assay method for total folate has proved its efficacy over many years. Plasma and serum samples can be assayed directly. Red blood cell (or whole-blood) samples require little manipulation. Although the short hydrolysis step that the process requires for red blood cell folate can result in some interconversion of one-carbon forms, all forms promote similar growth. The assay is relatively inexpensive and does not require sophisticated instrumentation. The composition of the medium is well defined, and researchers can prepare the medium

in a laboratory or obtain it from commercial sources. Bacterial growth is normally monitored by light transmission (actually light scattering) but can also be measured with the use of a pH meter because lactic acid bacteria produce lactate, and the medium's pH decreases from 6.1 to 4.7 at full growth. Because this organism produces energy by anaerobic metabolism, a shaking incubator is not required.

The *L. casei* assay procedure was further simplified  $\approx 20$  y ago with the introduction of a 96-well microplate technique (18–20). The assay's major experimental drawback is the need to maintain aseptic conditions because the growth medium is very rich. In some instances, particularly in clinical settings, the presence of antibiotics in blood samples may interfere with the assay. Antibiotic (chloramphenicol)-resistant strains of *L. casei* were introduced a number of years ago to allow the use of this bacterium for routine clinical laboratories (20).

### ASSESSMENT OF FOLATE STATUS

Approximately 50 y ago Baker et al (21) developed *L. casei* assay methods for the measurement of folate status and clinical folate insufficiency. The blood folate values that this assay produced at that time still form the basis for our current assessment of folate status adequacy and were the basis for the current folate dietary reference intake.

In a classic study by Herbert (22), who fed an adult male subject a folate-depleted (and inadvertently potassium-depleted) diet, serum folate concentrations fell before any other indicator of deficiency. Serum folate concentrations fell after 3 wk on the diet; sustained neutrophil hypersegmentation occurred after 11 wk; red blood cell folate concentrations declined after 17 wk; and Herbert observed megaloblastic marrow, a red cell count drop, and a sustained increase in urinary formiminoglutamate (*see below*) after 19 wk. This and many other clinical and nutritional studies at around the same time (reviewed in 23) led to the concept that serum (or plasma) folate is an indicator of recent folate intake, and fasting concentrations are a better indicator of short-term status (23). Red blood cell folate reflects tissue stores because red blood cells only accumulate folate during erythropoiesis. Furthermore, red blood cell folate concentrations change slowly with changes in intake because of the red blood cell's 120-d life span.

These early studies suggested that a serum folate concentration  $<3$  ng/mL represented clinical deficiency, concentrations between 3 and 6 ng/mL were marginal, and 6–20 ng/mL was the normal range. These ranges were the basis for the World Health Organization's serum folate guidelines in 1972 (24). The lack of megaloblastic changes in subjects with red blood cell folate concentrations  $>140$  ng/mL also formed the basis for the definition of the estimated average requirement for folate in the current National Academy of Sciences dietary reference intakes (25).

### COMPETITIVE PROTEIN BINDING ASSAYS

Clinical laboratories and population studies have used competitive binding assays widely for folate status assays since the 1970s. In many cases, competitive binding assays have supplanted the use of microbiological assays, primarily because of their ease of use and availability in commercial kit form. The initial competitive protein binding assays were based on serum or milk folate binders (26–29). Milk contains a soluble form of the

high-affinity folate binding protein (folate receptor). Unlike the microbiological assay methods, which measure the metabolic use of folate and consequently growth, the binding assays measure the competition between a labeled folate standard and unlabeled folate from biological extracts for a folate binder. The ability of folate in the biological sample to compete with the labeled folate standard depends on its relative affinities for the binding protein.

Folic acid has higher affinity than 5-methyl-THF for folate binding proteins, and the affinity of formyl-THF derivatives is somewhat lower. An early comparison of multiple commercial kits (30) showed differences in the response to different folate monoglutamate standards and folylpolyglutamate standards. With some kits, folylpolyglutamates bound with significantly higher affinity than monoglutamates and thus appeared to contain falsely high folate concentrations.

Accurate red blood cell folate measurements require complete conversion of red cell folate to the monoglutamate derivative. The different affinities of various monoglutamates for the binder are not a problem if 5-methyl-THF is the predominant one-carbon form in serum and red blood cells. However, the different affinities of various monoglutamates for the binder is a problem if significant concentrations of other one-carbon forms are present, such as in the red blood cells of methylenetetrahydrofolate reductase 677C  $\rightarrow$  T subjects (6). Most kits use labeled folic acid as the competitive binder, and some kit developers have tried to adjust the assay conditions (eg, pH) to try to equalize the affinity of folic acid and 5-methyl-THF. However, in general, the serum and red blood cell folate concentrations measured by these kits are significantly lower than the concentrations obtained from microbiological assays (31, 32), which possibly reflects the higher affinity of the folic acid standard. For this reason, manufacturers sometimes ask individual clinical laboratories to set their own concentrations from their experience for inadequate folate status. One manufacturer decreased the actual concentration of its folic acid standard by 30% from the concentration on the label so that the results with its kit agreed with the results from microbiological assays.

Generally, the range of folate concentration that laboratories can measure with these assays is 2–20 ng/mL. Although this range covers the lower expected serum folate concentrations, in an age of folate fortification many serum samples contain much higher concentrations than 20 ng/mL. Assays sometimes require diluted serum samples, and all red cell assays require considerable dilution because of their much higher folate concentrations. Unlike with microbiological assays, matrix effects are very marked with these binding protein kits and they produce significant differences in values, which depends on whether laboratories dilute samples (and standards) with buffer, human serum albumin, or "folate-free" serum or plasma (30, 32). Presumably, the matrix (pH, ionic strength, and so forth) influences the affinity of folates for the binder.

Over the past few years, the major kits that laboratories have used have been the Bio-Rad (Hercules, CA) Quantaphase II radioimmunoassay and the Abbott (Abbott Park, IL) AxSYM Folate ion capture radioimmunoassay. A number of NHANES cycles (33) have used the Bio-Rad kit. However, the manufacturer no longer produces this kit, which makes comparisons of earlier NHANES data with data from future NHANES cycles problematic. This issue shows a potential concern that can arise with the use of proprietary methods.

Comparison of the lower values for serum and red cell folate that laboratories obtain with these binding assay kits with the values with the *L. casei* assay also makes interpretation of folate status and adequacy of populations problematic because *L. casei* assays form the basis for current guidelines. Comparisons of kits from different manufacturers have also indicated wide variations between kits in the concentrations of serum and red blood cell folate that they identify (32).

### FUNCTIONAL INDICATORS OF FOLATE STATUS

Researchers have used a number of functional indicators of folate status over the years, which include plasma or urinary formiminoglutamate, aminoimidazole carboxamide riboside, and plasma homocysteine; elevations of these indicators reflect impaired one-carbon metabolism (1–3). Of these indicators, only homocysteine has received much traction. Although the levels of these indicators can change quite dramatically at the very lowest end of the folate status spectrum, they have little value in the definition of differences in the folate status of individuals in the low-normal to normal or even high folate status range because of a lack of sensitivity in this range. None of these indicators is specific for folate status because they can be elevated in vitamin B-12 deficiency and, in the case of homocysteine, vitamin B-6 and riboflavin deficiency (34).

### NEWER METHODOLOGIES

Mass spectrometry methods for the assay of folate derivatives have been reviewed by Pfeiffer et al (32, 35). These methods, which are still in the developmental stage, have the potential advantage of allowing the measurement of individual folate species, such as the major derivatives, in serum and red cells, as well as of relatively minor species, such as unmetabolized folic acid. This advantage is likely to become even more important in the future as more information on genetic polymorphisms that affect nutritional status and folate distributions becomes available. Because of fortification of the food supply with folic acid, concerns about folate status have shifted from the detection of folate deficiency in populations toward possible adverse effects of high folate intake. Mass spectrometry methods potentially allow the monitoring of the effects of high folic acid intake, such as the concentrations of folic acid in blood.

Experience with previous methodologies informs us about where some of the methodologic problems are likely to lie. Although theoretically every individual folate derivative would be accessible, so many potential derivatives exist that the capture of every one would be difficult, and a total folate measure would require the summation of all derivatives. Summation of the parts, as differential microbiological assays have shown, introduces additional error. With red blood cell folate, it may be necessary to hydrolyze polyglutamates to the monoglutamates to simplify the mixture, and incomplete hydrolysis would decrease recoveries.

Chemical work-up and any derivatization procedures will probably lead to loss of some species and interconversion of others. Laboratories can account for losses by the addition of internal standards for the folate derivatives of interest. However, adjustment for the extent of interconversion of folate forms is more difficult. Interconversion of species can lead to loss of some forms and increases in others. For example, if folic acid is of interest, it

will be necessary to ensure that other folate derivatives do not generate folic acid *in situ* during the procedure.

Bagley and Selhub (36) have described an intriguing method that involves purification of folates by binding and elution from an immobilized folate binding protein column and HPLC separation of the individual folate forms in combination with electrochemical detection. The use of various voltages for the detector allowed the detection and measurement of individual classes of one-carbon forms of folate without complete separation of all forms. Studies have not yet validated this method extensively, particularly with regard to the stability of individual folate forms and the extent of their interconversion during the purification and separation procedures.

### CONCLUDING REMARKS

Despite its age, the *L. casei* microbiological assay procedure for the measurement of total folate in blood samples remains the simplest and most easily interpretable method for assessment of overall folate status in large population survey groups such as NHANES. Cutoff values for folate status sufficiency are well established and time trends are easily discerned. Comparisons with data obtained with other population groups, both large and small, are straightforward. The mass spectrometry methods for folate assay, which are currently under development, show much promise. These methods will need further validation and standardization to ensure that consistent data are obtained, which can form the basis for the establishment of new ranges for folate status sufficiency.

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